A1 cont

exploited in protein identification, but are not as widely used as immunorecognition. Although not biospecific, immobilized metal affinity chromatography (IMAC) is yet another affinity method that recognizes a specific structural element of polypeptides (J. Porath et al., *Nature* 258: 598-599 (1975)).

Please replace the paragraph at page 3, lines 15-29, with the following rewritten paragraph. Per 37 C.F.R. §1.121, this paragraph is also shown in Appendix A with notations to indicate the changes made.

A2

The fractionation approach to protein identification in mixtures is often more lengthy because analytes must be purified sufficiently to allow a detector to recognize specific features of the protein. Properties ranging from chemical reactivity to spectral characteristics and molecular mass have been exploited for detection. Higher degrees of purification are required to eliminate interfering substances as the detection mode becomes less specific. Since no single purification mode can resolve thousands of proteins, multidimensional fractionation procedures must be used with complex mixtures. Ideally, the various separation modes constituting the multidimensional method should be orthogonal in selectivity. The two-dimensional (2D) gel electrophoresis method of O'Farrell (*J. Biol. Chem.* 250:4007-4021 (1975)) is a good example. The first dimension exploits isoelectric focusing while the second is based on molecular size discrimination. At the limit, 6000 or more proteins can be resolved. 2D gel electrophoresis is now widely used in proteomics where it is the objective to identify thousands of proteins in complex biological extracts.

Please replace the paragraph at page 27, lines 9-15, with the following rewritten paragraph. Per 37 C.F.R. §1.121, this paragraph is also shown in Appendix A with notations to indicate the changes made.

Tryptophan is present in most mammalian proteins at a level of <3%. This means that the average protein will yield only a few tryptophan containing peptides. Selective derivatization of tryptophan has been achieved with 2,4-dinitrophenylsulfenyl chloride at pH 5.0 (M. Wilcheck et al., Biochem. Biophys. Acta 278:1-7 (1972)). Using an antibody directed against 2,4-dinitrophenol, an immunosorbant was prepared to select peptides with this label. The advantage of tryptophan selection is that the number of peptides will generally be small.

A3

Please replace the paragraph at page 31, lines 5-27, with the following rewritten paragraph. Per 37 C.F.R. §1.121, this paragraph is also shown in Appendix A with notations to indicate the changes made.

After peptides of interest are detected using mass ~~spectrometry;~~ the protein from which a peptide originated is determined. In most instances this can be accomplished using a standard protocol that involves scanning either protein or DNA databases for amino acid sequences that would correspond to the proteolytic fragments generated experimentally, matching the mass of all possible fragments against the experimental data (F. Hsieh et al., Anal. Chem. 70:1847-1852 (1998); D. Reiber et al., Anal. Chem 70:673-683 (1998)). When a DNA database is used as a reference database, open reading frames are translated and the resulting putative proteins are cleaved computationally to generate the reference fragments, using the same cleavage method that was used experimentally. Likewise, when a protein database is used, proteolytic cleavage is also performed computationally to generate the reference fragments. In addition, masses of the reference peptide fragments are adjusted as necessary to

A4

reflect derivatizations equivalent to those made to the experimental peptides, for example to include the exogenous affinity tag. The presence of signature peptides in the sample is detected by comparing the masses of the experimentally generated peptides with the masses of signature peptides derived from putative proteolytic cleavage of the set of reference proteins obtained from the database. Software and databases suited to this purpose are readily available either through commercial mass spectrometer software or the Internet. Optionally, the peptide databases can be preselected or reduced in complexity by removing peptides that do not contain the amino acid(s) upon which affinity selection is based.

A4

Please replace the paragraph at page 61, lines 1-3, with the following rewritten paragraph. Per 37 C.F.R. §1.121, this paragraph is also shown in Appendix A with notations to indicate the changes made.

A5

2. Cysteine can be derivatized with an affinity tagged maleimide. Normal and deuterium labeled tags are mixed so that tagged species are easily identified in the MALDI spectrum as a doublet that is three mass units apart.

Please replace the paragraph at page 62, lines 1-2, with the following rewritten paragraph. Per 37 C.F.R. §1.121, this paragraph is also shown in Appendix A with notations to indicate the changes made.

A6

3. Cysteine can alternatively be derivatized with 2,4-dinitrobenzyl chloride. Conditions: pH 5, 1 hour, room temperature.

Please replace the paragraph at page 67, lines 5-19, with the following rewritten paragraph. Per 37 C.F.R. §1.121, this paragraph is also shown in Appendix A with notations to indicate the changes made.

RPC

↑

select for cysteine second (exogenous affinity ligand)

↑

RPC ← select for glycosylation, phosphopeptides or histidine first (endogenous affinity ligands)

↑

Protein → reduced protein → alkylated protein* → tryptic peptides

↓

| | | |
|---|---|---|
| affinity select for cysteine first (exogenous affinity ligand) | – | secondary affinity labeling (tryptophan, methionine or tyrosine) |
| ↓ | | ↓ |
| affinity select for tryptophan, methionine or tyrosine second (exogenous affinity ligands) | | select for tryptophan, methionine, or tyrosine first (exogenous affinity ligands) |
| ↓ | | ↓ |
| RPC | | RPC |

Please replace the paragraph at page 70, lines 10-19, with the following rewritten paragraph. Per 37 C.F.R. §1.121, this paragraph is also shown in Appendix A with notations to indicate the changes made.

Data are presented that suggest proteins may indeed be quantified as their signature peptides by using isotopically labeled internal standards. Signature peptides generated by trypsin digestion have a primary amino group at their amino-terminus in all cases except those in which the peptide originated from the blocked amino-terminus of a protein. The specificity of trypsin cleavage dictates that the C-terminus of signature peptides will have either a lysine or arginine (except the C-terminal peptide from the protein) and that in rare cases there may also be a lysine or arginine adjacent to the C-terminus. Primary amino groups of peptides were acylated with *N*–hydroxysuccinimide.